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Regulation of Mammary Differentiation by Extracellular Matrix Involves Protein-tyrosine Phosphatases*

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Extracellular matrix and growth factors cooperate to regulate signaling pathways and gene transcription in adherent cells. However, the mechanism of extracellular matrix signaling is poorly defined. In mammary gland, the expression of milk protein genes is controlled by cross-talk between signals derived from the basement membrane protein, laminin, and the lactogenic hormone, prolactin. Signals from basement membrane are transduced by β_1 integrins and are required for prolactin to activate DNA binding of the milk protein gene transcription factor, Stat5. Here we show that basement membrane is necessary for tyrosine phosphorylation of the prolactin receptor and thus directly affects cytokine signaling and differentiation at the level of the plasma membrane. Prolactin does not induce tyrosine phosphorylation of its receptor, Jak2, or Stat5 in nondifferentiated breast epithelia cultured on collagen I, and we show that this is due to a vanadate-sensitive activity that inhibits the prolactin pathway. We suggest that protein-tyrosine phosphatases are novel targets for regulation by extracellular matrix and in mammary cells represent an additional control to the requirement of integrins for milk protein production.

Cell behavior is controlled by a network of signals derived from growth and differentiation factors as well as from the local cellular environment. These signals are interpreted by appropriate receptors and converted into intracellular pathways that modulate transcriptional or post-transcriptional events. Migration, proliferation, survival, and differentiation are strongly influenced by cell interactions with the extracellular matrix (ECM)[1] (1). For example, integrins determine the activity of both Ras-mitogen-activated protein kinase and phosphatidylinositol 3′-kinase mediated growth factor responses, and cell-ECM interactions contribute to cyclin activation thereby regulating cell cycle entry (2–6). However, the mechanism for this ligand-induced signaling cross-talk has not been established. Many signal transduction pathways, including those conveyed by ECM-integrin interactions are controlled by protein-tyrosine kinases (PTKs) (7). Homeostasis of signaling requires negative regulation through protein-tyrosine phosphatases (PTPs) (8, 9), and these enzymes are therefore of potential significance in the control of ECM signaling.

In the breast, epithelial cell interactions with basement membrane (BM) control the prolactin-dependent expression of milk protein genes (10, 11). Both laminin-1 and β_1 integrins are required for differentiation of mammary cells (12, 13) and, together with prolactin, direct milk protein gene transcription. The molecular details of the prolactin signaling pathway were first described in the rat lymphoma cell line, Nb2, which requires prolactin for proliferation. Ligation of the prolactin receptor (PrlR) results in induction of the associated PTK activity, Jak2 (14–16). This leads to activation of Stat transcription factors that recognize specific DNA sequence motifs in the promoters of early response genes implicated in proliferation (17, 18). Prolactin triggers differentiation through a similar pathway, and in cells transfected with PrlR, Stat5, and β-casein reporter vectors, prolactin induces transcription from the β-casein promoter via activation of endogenous Jak2 and ectopically expressed Stat5 (19).

Mammary epithelial cells are adherent and, in contrast to Nb2 cells which grow in suspension, require cell-BM interactions to propagate prolactin signals. Using primary cultures of mouse mammary epithelial cells, we recently demonstrated that BM contributes to prolactin-dependent transcription of milk protein genes by regulating the DNA binding activity of Stat5 (20). Only cells cultured on BM and stimulated with prolactin were able to induce Stat5 activity and produce milk proteins, whereas cells on collagen I did not respond to hormone because they showed no Stat5 activity or milk protein synthesis after prolonged culture with prolactin. These results showed that mammary cells exhibit a strong dependence on both prolactin and BM for Stat 5 DNA binding activity, and we argued that this provided a molecular mechanism to explain the regulation of milk protein gene transcription by the ECM (20).

In this paper we examine whether BM regulates Stat5 DNA binding activity directly or through a control on its upstream signaling components Jak2 and PrlR. In short term experiments where Stat5 activity was induced after 15 min of hormone treatment, we found that BM was required for prolactin signaling both at the level of its receptor as well as Jak2. Moreover, the lack of differentiation in mammary cells cultured on collagen I was additionally due to a vanadate-sensitive activity that inhibited phosphorylation of PrlR, Jak2, and Stat5. Thus, in the absence of correct ECM signals PTPs appear to inhibit cytokine-triggered second messengers, providing a novel paradigm for the mechanism of ECM signaling.

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[1] The abbreviations used are: ECM, extracellular matrix; BM, basement membrane; EMSA, electromobility shift assay; PrlR, prolactin receptor; PTP, protein-tyrosine phosphatase; PTK, protein-tyrosine kinase.

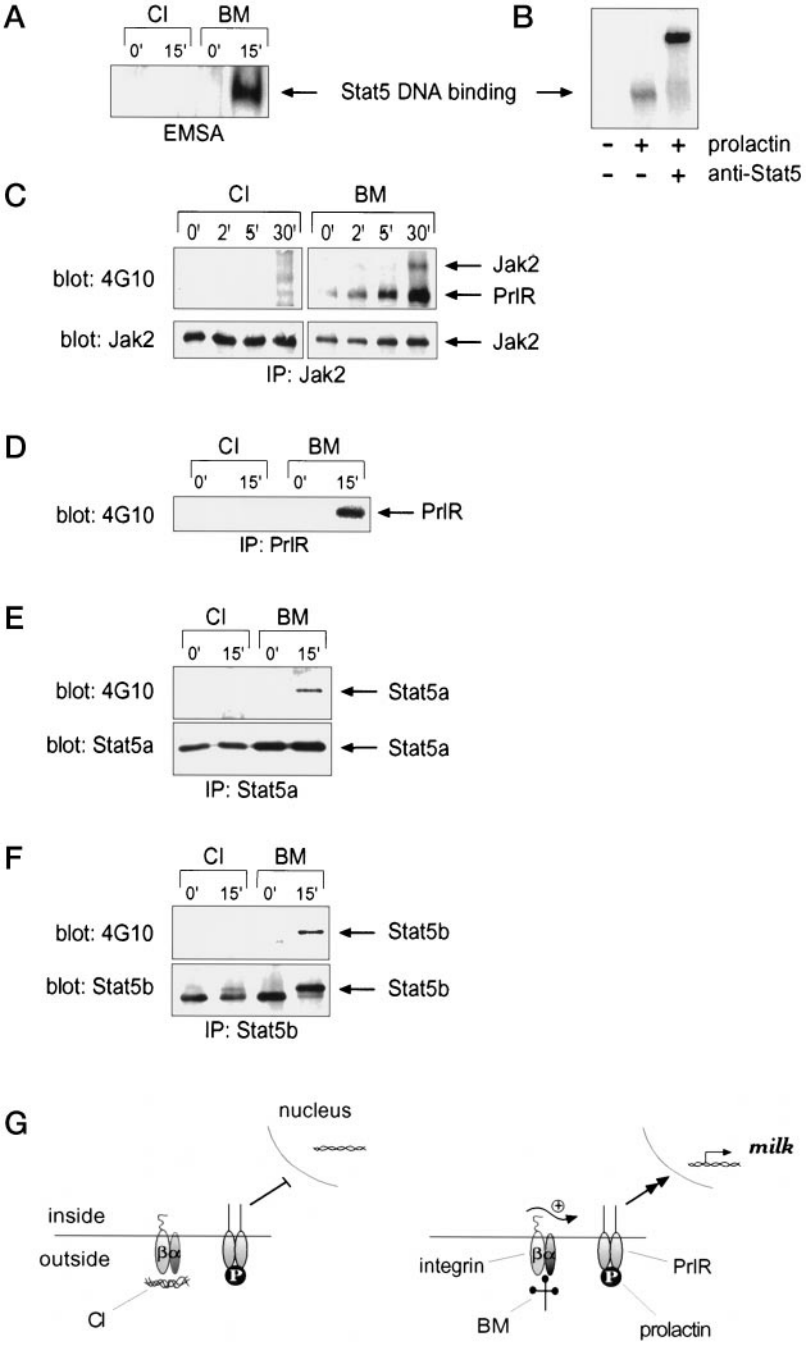

FIG. 1. **Prolactin signaling requires cross-talk with an appropriate ECM.** *A–F*, primary mouse mammary epithelial cells were cultured on collagen I (*CI*) or BM in differentiation medium, incubated in fresh medium containing 150 nM prolactin for the indicated times, and harvested. *A*, nuclear extracts were examined by EMSA using an oligonucleotide recognized by Stat5 (*25*). *B*, to confirm the presence of active Stat5, EMSA of nuclear extracts from BM cultured cells was performed with (+) or without (−) anti-Stat5 antibody. *C–F*, after stimulating with prolactin for the indicated times, the cells were lysed and immunoprecipitated (*IP*) with antibodies to Jak2 (*C*), PrlR (*D*), Stat5a (*E*), and Stat5b (*F*) using the lysates from 2×10^7, 5×10^6, 3×10^6, and 5×10^6 cells, respectively. After separation by 6.25% SDS-polyacrylamide gel electrophoresis, precipitated proteins were analyzed by immunoblotting with antibodies for phosphotyrosine (4G10) or the appropriate precipitating antibody. As expected, anti-Jak2 immunoprecipitated both Jak2 and the PrlR. Note that prolactin induces an apparent increase in the molecular weight of Stat5b but not that of Stat5a. We have not been successful in Western blotting prolactin receptor in mouse mammary epithelia with the available reagents, however, the results presented in Fig. 4 indicate that it is present in cells cultured on collagen and is potentially able to respond to prolactin signaling. *G*, prolactin (*P*) is unable to trigger signals that lead to milk protein gene expression in mammary epithelial cells cultured on collagen I (*left*) but activates signaling in cells that interact with laminin-rich BM (*right*). Because integrins are required for milk protein synthesis (*13*), we propose that laminin provides a positive signal for the prolactin cassette to be triggered.

EXPERIMENTAL PROCEDURES

Cell Culture–Mammary epithelial cells were isolated from 14.5–18.5-day pregnant ICR mice and established in culture at a density of 2.5–5 $\times$ 10^5 cells/cm as described (21). Cells were plated on dishes coated with either collagen I or laminin-rich BM matrix (12) and cultured for 48–72 h in Ham's F-12 medium (Sigma-Aldrich Co. Ltd., Poole, UK) containing 10% heat-inactivated fetal calf serum (Advanced Protein Products, Brierley Hill, UK), 10 ng/ml epidermal growth factor (Promega Corp., Southampton, UK), 1 mg/ml fetuin, 880 nM insulin, and 2.8 nM hydrocortisone. The cultures were washed extensively, and the medium was changed to differentiation medium (Dulbecco's modified Eagle's medium/Ham's F-12 medium (Life Technologies Ltd., Paisley, Scotland) containing 880 nM insulin, 2.3 nM hydrocortisone) for a further 24–72 h before stimulating with 150 nM prolactin for the prescribed times.

Protein Analysis–Cells were washed in ice-cold phosphate-buffered saline containing 1 mM sodium orthovanadate and then extracted into lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM pepstatin A, 10 mM leupeptin and aprotinin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 20 mM β-glycerophosphate, and 10 μM ammonium molybdate). After clearing the detergent-insoluble proteins by centrifugation, lysates from equal numbers of cells from the collagen I or BM cultures were immunoprecipitated with anti-Jak2 antibody (Upstate Biotechnology Inc., Lake Placid, NY), anti-prolactin receptor antibody 46 (22), or anti-Stat5a and anti-Stat5b antibodies (23) followed by protein A-Sepharose (Zymed Laboratories Inc., South San Francisco, CA) before separation by 6.25% SDS-polyacrylamide gel electrophoresis. After transfer to Immobilon P membrane (Millipore Ltd., Watford, UK), phosphorylated proteins were revealed with the anti-phosphotyrosine antibody, 4G10 (Upstate Biotechnology Inc.) followed by enhanced chemiluminescence using an ECL kit (Amersham International plc, Little Chalfont, UK). Blots were stripped according to the Amersham protocol and reprobed with precipitating antibody.

In some experiments where vanadate was used, sodium pervanadate was freshly prepared by mixing equal volumes of 20 mM sodium orthovanadate and 20 mM hydrogen peroxide for 30 min at room temperature. 100 units catalase was added to destroy any unreacted hydrogen peroxide, and the sodium pervanadate was kept on ice. Cells were incubated in fresh medium for 2 h before changing to differentiation

medium containing 25 μM sodium pervanadate for 45 or 120 min. 150 nM prolactin was added for specified times prior to harvesting.

Electrophoretic Mobility Shift Assays (EMSA)–Primary cultures of mammary epithelial cells from pregnant ICR mice were harvested by trypsinization. Cell pellets were snap frozen in liquid N_2, and nuclear extracts were prepared as described (24). The total protein concentration was estimated by the Pierce BCA protein assay (Pierce and Warriner (UK) Ltd., Chester, UK), and 4 μg extract was incubated with 0.5 ng of end-labeled double-stranded oligodeoxynucleotide (*i.e.* the Stat5 STM site, 5′-GATTCCGGGAACCGCGT; Ref. 25) as described (26) excluding the addition of single-stranded competitor oligodeoxynucleotide. The nuclear extract was added as the final component of the reaction. In supershift assays, nuclear extracts were incubated for 30 min at room temperature with antibodies to Stat1 (M22), Stat 3 (C20), Stat5a (L20), or Stat5b (C17; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) before addition of DNA.

RESULTS

To determine the point of intersection of the BM stimulus with the prolactin cassette, we used a short term assay where the prolactin signaling proteins were rapidly activated in primary cultures of mouse mammary epithelial cells. In this assay, the transcription factor Stat5 was activated 15 min after hormone treatment in cells contacting a reconstituted BM but not in those on the stromal matrix, collagen I (Fig. 1, *A* and *B*). The extent of tyrosine phosphorylation in the prolactin signaling proteins was examined, and all the components were found to be phosphorylated in response to hormone in cells cultured on BM but not in cells on collagen (Fig. 1, *C–F*). In addition, prolactin treatment resulted in an apparent increase in the molecular weight of Stat5b (27). Thus, in adherent mammary cells, there is an ECM-specific component of prolactin signaling that acts at the level of the PrlR. Integrins are required for prolactin-dependent milk protein synthesis (13), indicating that the differentiation signal from BM positively influences the prolactin cassette (Fig. 1*G*), either directly through receptor clustering (28) or indirectly through an additional pathway.

The lack of signaling in mammary cells cultured on collagen I was not simply due to a delayed prolactin response, because Jak2 phosphorylation and Stat5 DNA binding activity were not detected in cells treated for up to 2 days with prolactin.[2] However, in cultures on BM, we noted that the early signaling events were transient. Tyrosine phosphorylation of Jak2 (Fig. 2*A*) and co-immunoprecipitated PrlR (Fig. 2, *A* and *B*) and of Stat5a and Stat5b (Fig. 2, *C* and *D*) as well as Stat5 DNA binding activity (Fig. 2*E*) occurred rapidly before diminishing to basal levels within 60–120 min. To determine whether this down-regulation of the prolactin cassette shortly after its initial activation was controlled by a PTP activity (29), mammary epithelial cells cultured on BM were incubated with low concentrations (25 μM) of the PTP inhibitor sodium pervanadate (30). After treating cells with prolactin for 120 min, Stat5a and Stat5b retained the maximum level of phosphorylation that had been achieved in vanadate-free cultures (Fig. 2, *C* and *D*). The protein levels remained constant throughout the experimental period, indicating that they were first phosphorylated in response to hormone but were subsequently dephosphorylated. Thus, the prolactin-induced phosphorylation of Jak2, PrlR, and Stat5 is transient, and once activated the pathway subsequently comes under negative control by a PTP that reduces the signal 30–60 min after initiation.

We then asked whether the initial events leading to activation of Stat5 were also influenced by vanadate-sensitive mechanism. Mammary cells cultured on BM were treated with sodium pervanadate for 30 min and then incubated for an additional 15 min with or without prolactin. PTP inhibitor treatment led to hormone-independent activation of the prolactin signaling cassette including the phosphorylation of Jak2, PrlR, Stat5a, and Stat5b (Fig. 3*A*) as well as the induction of Stat5 DNA binding activity (Fig. 3*B*), which was confirmed by supershift assays (Fig. 3*C*). Prolactin triggers Jak-Stat signaling through receptor dimerization, which leads to phosphorylation of Jak2 (14–16), but our data now show that PTP inhibition also contributes to Jak2 activation. This suggests that a PTP and a kinase are normally functionally associated to suppress prolactin signaling, but this can be overcome by either the natural ligand, prolactin, or by artificial inhibitors of PTPs (Fig. 3*D*). Although vanadate resulted in ligand-independent Stat5 DNA binding, milk proteins were not synthesized even in the presence of prolactin[3]; an additional PTP may therefore be required (31) for Stat5 to induce milk protein gene transcription (19), or alternatively, Stat1 and Stat3, which were also activated under these conditions (Fig. 3*C*), may compete for Stat5 DNA binding sites.

Our observation that PTPs may be involved with initial events of prolactin signaling suggested that PTPs might also be implicated in the lack of response to prolactin in mammary cells cultured on collagen I (Fig. 1). Cells on collagen were treated similarly with sodium pervanadate. Now hormone stimulation led to the phosphorylation of Jak2, PrlR, and Stat5 proteins (Fig. 4*A*). Thus, in contrast to cells on BM, prolactin

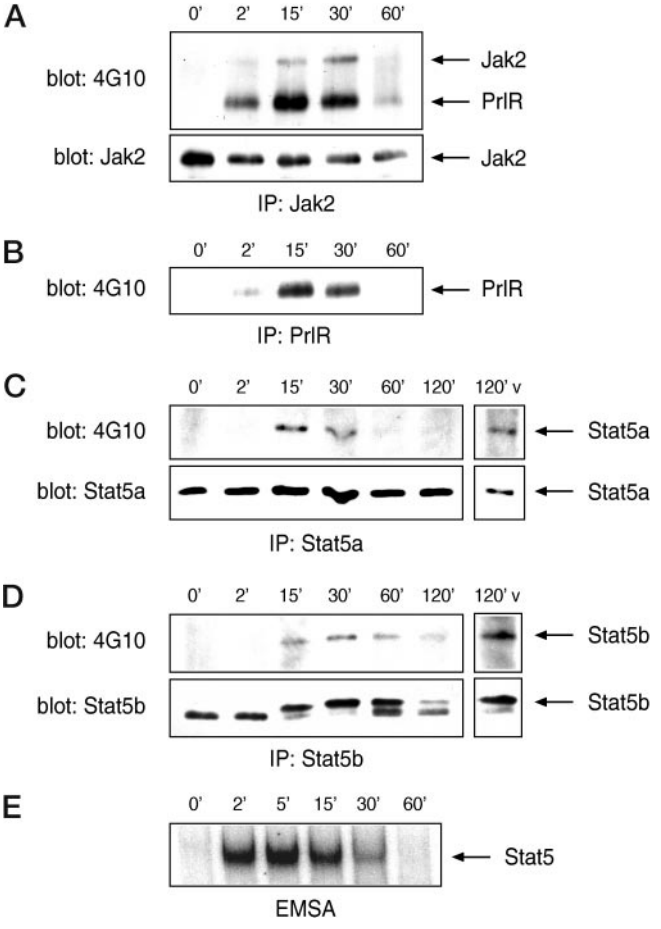

FIG. 2. **Activation of the prolactin signaling pathway is transient.** *A–E*, primary cells cultured on BM were exposed to 150 nM prolactin for the indicated times. Stat5a and Stat5b cultures were also treated with 25 μM sodium pervanadate for 120 min (*120′v*). *A–D*, lysates were immunoprecipitated (*IP*) with Jak2 (*A*), PrlR (*B*), Stat5a (*C*), and Stat5b (*D*) antibodies using 2×10^7, 3.5×10^6, 1×10^6, and 3×10^6 cells, respectively, and analyzed by immunoblotting as in Fig. 1. *E*, EMSA of nuclear extracts using an oligonucleotide recognized by Stat5.

[2] G. M. Edwards and C. H. Streuli, unpublished data.

[3] F. H. Wilford and C. H. Streuli, unpublished data.

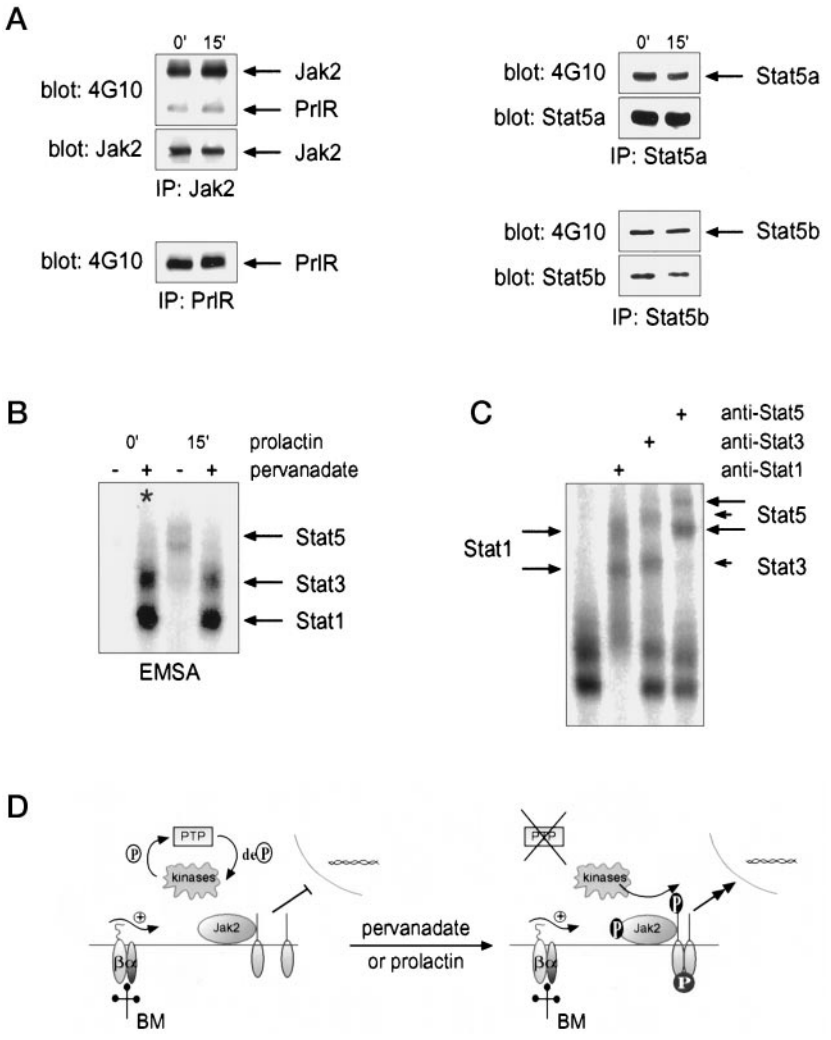

FIG. 3. **Prolactin triggers its signaling cassette by altering PTP/kinase balance.** *A–C*, primary mouse mammary epithelial cells cultured on BM were treated with 25 μM sodium pervanadate for 30 min. After stimulating with 150 nM prolactin for a further 15 minutes, the cells were harvested. *A*, lysates from cells on BM were immunoprecipitated (*IP*) with antibodies against Jak2, PrlR, Stat5a, and Stat5b (using 5, 4, 2, and 5×10^6 cells/precipitation respectively) and immunoblotted with 4G10 or the appropriate precipitating antibody as in Fig. 1. *B*, EMSA was performed on nuclear extracts from cells which had (+) or had not (−) been treated with sodium pervanadate and treated with prolactin for 15 min. In some experiments when the gels were electrophoresed for longer, the Stat5 binding activity resolved into two bands. *C*, to show that pervanadate induced Stat1, Stat3, and Stat5 activity in the absence of prolactin, EMSA was performed on the nuclear extract marked with an *asterisk* in *B*, in the presence of the indicated anti-Stat1, anti-Stat3, or a mixture of anti-Stat5a and anti-Stat5b antibodies. *Arrows* indicate the positions of supershifted Stats (large and small left-pointing arrows indicate Stat5 and Stat3 supershifts, respectively). *D*, treatment of mammary cells cultured on BM with pervanadate leads to ligand-independent activation of prolactin signaling, as demonstrated in *A*. In the absence of ligand, a PTP keeps the pathway in check by acting directly on Jak2 or an additional unidentified kinase (as shown). Prolactin alters the overall PTP/kinase balance allowing commencement of signaling.

signaling is inhibited in cells on collagen I by a PTP that cannot be inactivated by the hormone alone. We noted that the pathway could not be stimulated fully by pervanadate in cells cultured on collagen I. Although Stat5b was tyrosine phosphorylated, it was only partially altered in molecular weight (Fig. 4*A*), and no Stat5 DNA binding activity (Fig. 4*B*) or milk protein synthesis[3] was observed even in the presence of prolactin. This indicates that additional factors, for example cell geometry (32, 33), contribute to differentiation. Together the data show that one role for BM in mammary differentiation is to regulate the PTP/PTK balance, thereby allowing prolactin to trigger its signaling cassette (Fig. 4*C*).

DISCUSSION

Two important conclusions can be derived from this study. First, one mechanism by which BM regulates milk protein gene transcription is by permitting prolactin to trigger the intracellular phosphorylation cascade, which leads to Stat5 transcription factor activation. Second, there is a vanadate-sensitive inhibition of prolactin signaling when mammary cells are cultured on collagen, indicating an involvement of PTPs in cell regulation by ECM.

Cross-talk between BM and Prolactin Signaling–In earlier studies we demonstrated that Stat5 DNA binding activity could only be detected in mammary cells cultured on BM, not in cells on collagen (20), and therefore argued that the previously established role for BM on milk protein synthesis (12, 34) was exerted at the level of transcription factor activation. We now show that BM signals interact with the prolactin pathway upstream of Stat5 at the plasma membrane.

These results indicate that in adherent mammary epithelial cells, the prolactin pathway is not controlled simply by the presence of an endogenous cytokine as it is in hematopoietic cells (14–18), but rather it is under positive regulation from both the cytokine and a specific type of ECM. Because β_1 integrins are essential for milk protein synthesis in mammary epithelial cells (13), the BM-integrin interaction may result in the formation of a physical complex with PrlR signaling components as is the case with $\alpha v\beta 3$ integrin and the platelet-derived growth factor receptor (35), or alternatively there may be indirect cross-talk between integrin and cytokine receptors.

It has been suggested that there is a hierarchical accumulation of many different components within an integrin signaling complex (36). Indeed, adhesion complexes containing integrins and signaling proteins such as $pp125^{FAK}$, $pp60^{c\text{-}Src}$, phospholipase C-γ, Rho, Ras, and mitogen-activated protein kinase have been identified (28, 37), and moreover integrins have been found to be associated with IRS-1 (38), Shc (39), and platelet-derived growth factor receptor (35). A model has therefore emerged whereby integrins assemble appropriate signaling molecules into a complex, which can respond to growth factor and cytokine stimulation. This provides one potential mechanism for ECM involvement in proliferation, where growth factors and ECM cooperate to trigger the signaling cassettes that ultimately result in cyclin D-cdk4/6 and cyclin E-cdk2 complex activation (6) and progression through the cell cycle (2, 35).

The data presented here indicate that ECM also controls

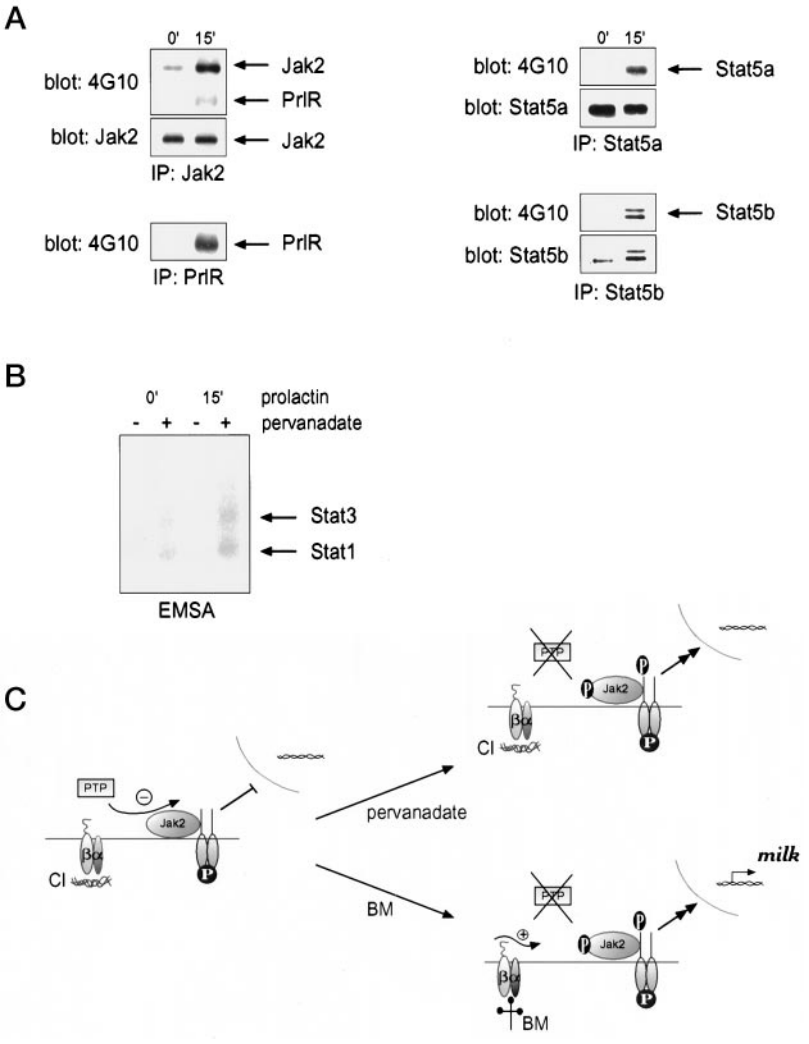

FIG. 4. **A PTP prevents prolactin signaling in cells cultured on collagen I.** *A–B*, primary mouse mammary epithelial cells cultured on collagen I were treated with 25 μM sodium pervanadate for 30 min and analyzed as in Fig. 3. Equivalent amounts of protein for immune precipitates (*IP*) and EMSA were used, and gels were exposed for the same times as in Fig. 3. Prolactin now induced phosphorylation of its signaling proteins, but Stat5 DNA binding activity was not detected. *C*, a PTP blocks ligand-dependent activation of prolactin signaling on collagen I. Treatment of mammary epithelial cells cultured on collagen I with pervanadate results in ligand-dependent phosphorylation of early components of the prolactin cassette, as demonstrated in *A*. Thus, a PTP blocks prolactin signaling in cells cultured on inappropriate substrata, but it can be inactivated by culture on a laminin-rich BM.

cytokine-triggered pathways regulating differentiation. By analogy with the proliferation model, the differentiation signal from BM may arise from interactions within multiprotein signaling complexes containing both integrin and prolactin receptors, and one of our current goals is to determine whether this is the case.

PTPs Regulate Activation of Prolactin Signaling—Regulation of homeostasis within the cell by PTKs is mediated by PTPs (8, 9). The involvement of PTPs in cell signaling pathways has frequently been assessed by the use of vanadate (30, 40–42), because there are virtually no inhibitory reagents currently available for specific members of the PTP family. Our results indicate that there are at least three vanadate-sensitive steps in prolactin signaling in mammary cells.

First, the down-regulation of Stat5 phosphorylation 120 min after prolactin treatment was inhibited by vanadate. The PTP, SHP-1, inactivates the homologous erythropoietin signaling pathway by binding to the erythropoietin receptor shortly after initial activation (29). In addition, SHP-1 binds tyrosine phosphorylated Stat5 after growth hormone activation and mediates its dephosphorylation (43). Thus, our results may reflect a similar involvement of PTP in attenuating prolactin-triggered signaling in mammary cells.

Second, the initial phosphorylation events in prolactin signaling were independent of ligand following short treatments with low concentrations of vanadate. Given that prolactin is currently perceived to activate its signaling pathway through the PTK, Jak2 (44), our results suggest that Jak2 may normally be under negative regulation by a PTP. One action of prolactin-mediated ligation of its receptor may therefore be to alter the balance of a PTP-Jak2 cycle rather than merely activate the kinase.

Third, in mammary cells cultured on collagen, vanadate enabled prolactin to trigger the early phosphorylation events in its signaling pathway. The implication is that in the absence of correct ECM signals, a PTP may inhibit cytokine-dependent signaling. This represents a novel aspect of ECM control on cell differentiation.

We do not yet know the identity of the putative PTP(s) involved with ECM signaling in mammary cells or indeed whether the regulation is through PTP-interacting proteins such as signal-regulatory proteins (45, 46). However, the results suggest that PTPs may contribute to selectivity of integrin responses for controlling differentiation. For example, the laminin-binding integrins $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$ are all expressed in mammary epithelial cells (47) but $\alpha2\beta1$ is preferentially used for interactions with collagen I, whereas $\alpha3\beta1$ and $\alpha6\beta1$ interact with laminin (48). Thus, a PTP might either be inactivated by a specific integrin-dependent cell interaction with BM, thereby promoting a phosphorylation cascade after ligand stimulation, or alternatively the PTP might not be recruited to BM-induced adhesion complexes.

An analogous situation may arise in the control of ECM interactions on other aspects of cell phenotype. For example, growth factors are unable to induce proliferation and promote survival in cells cultured in suspension (2, 3). Because the

inhibition of cell-ECM interactions in fibroblasts leads to increased PTP activity (49), the inability to respond to soluble factors may be partly due to activation of PTPs.

Early events in cell adhesion to ECM have been associated with altered tyrosine phosphorylation, which appears to be important for recruitment of cytoskeleton to adhesion complexes (7). Altered phosphorylation following vanadate treatment may therefore influence cytoskeletal organization and thereby affect prolactin receptor dimerization or other receptor proximal kinases involved in prolactin signaling. However, immunofluorescent examination of the actin- and tubulin-based cytoskeleton in mammary cells indicated that there was no disruption of the cytoskeleton following short term treatment with vanadate.[4] This contrasts with the action of cytochalasin D where the actin-containing structures in similar cultures were severely perturbed within 30 min.

PTPs have previously been shown to be involved in the regulation of signaling by cytokines (31, 29) and may also participate in cell-cell communication (50–52). We now propose that PTPs are targets for the ECM signals that regulate differentiation in mammary cells, possibly through direct interaction with proteins within adhesion complexes (53, 54).

Summary—Our work offers a new paradigm to explain cell regulation by ECM, in which signals from correct integrin-ECM interactions positively influence cytokine-induced differentiation, whereas PTP-mediated inhibition of cytokine signaling occurs when cells are in an inappropriate ECM environment.

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